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Differential Expression of the *KLK2* and *KLK3* Genes in Peripheral Blood and Tissues Samples of Iranian Patients with Prostate Cancer

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ABSTRACT

Background and Objectives: Prostate cancer is a highly prevalent malignancy with a high mortality rate in men. Many studies have investigated the diagnostic value of various genes involved in prostate cancer, but there is no data for *Kallikrein 2* (*KLK2*) and *Kallikrein 3* (*KLK3*) expression patterns among Iranian patients. Therefore, we aimed to evaluate the expression of these two genes in Iranian patients with prostate cancer.

Methods: In this case-control study, three groups consisting of healthy individuals, patients with benign prostatic hyperplasia (BPH) and patients with prostate cancer were studied. Peripheral blood samples were collected from all subjects, mRNA was extracted after cell lysis, and cDNA was synthesized. Real-time PCR was performed to assess gene expression levels relative to a reference gene (18s rRNA gene).

Results: The *KLK2* gene was overexpressed in patients with prostate cancer. *KLK2* expression differed significantly between the cancer patients and controls. Relative expression of the *KLK3* gene in the BPH group was higher than that in the control and cancer groups. However, we observed no significant difference in the expression of *KLK3* between the control and cancer subjects.

Conclusion: We demonstrate that analysis of *KLK2* expression is a favorable method of diagnosing prostate cancer and discriminating normal individuals from those with BPH or prostate cancer. We also found that the *KLK3* gene is highly overexpressed in individuals with BPH, which might indicate that this gene is not cancer-specific.

Keywords: Prostatic Neoplasm, Prostatic Hyperplasia, Kallikreins, Gene Expression.

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INTRODUCTION

Prostate cancer is the second leading cause of cancer death in developed countries (1, 2). It is estimated that about one out of every six men will be diagnosed with prostate cancer during his lifetime (3, 4). According to epidemiological studies, the prevalence of prostate cancer has been consistently increasing in many developed countries over the last few decades. In Iran, cancer is the third leading cause of death following cardiovascular diseases and traffic accidents (5, 6). A study in Tehran (Iran) reported that prostate cancer is the most prevalent type of cancer in men following gastric cancer. It is noteworthy to mention that the prevalence of prostate cancer is higher in Tehran than in Ardebil (Iran), Karachi (Pakistan), Izmir (Turkey) and Dubai (UAE) (7).

One of the methods for rapid detection of prostatic neoplasms is to examine the involved genes and their products. The human kallikrein genes *KLK2* and *KLK3* both have five coding exons and four introns and produce at least 13 and 5 types of mRNA transcripts, respectively (8, 9). These transcripts are generated because of alternative splicing or polyadenylation (8, 10, 11). *KLK3* is the most popular kallikrein gene associated with prostate cancer (12). It belongs to a family of 15 kallikreins most commonly known as prostate-specific antigen (PSA), a protein produced by both normal and malignant cells of the prostate gland (13). Studies on PSA expression revealed that this molecule is not prostate-specific and is in fact produced in other tissues, such as the breast (14).

KLK2 and *KLK3* are reported to show a homology degree of 80-85% (8). The expression of *KLK3* is increased in benign prostatic hyperplasia (BPH) and highly differentiated prostate cancer. However, it is decreased during prostate cancer progression (15, 16). For several years, PSA has been used as a tumor marker of choice despite its low specificity. In approximately 15% of men with serum PSA levels of less than 4 ng/ml, prostate cancer is diagnosed through prostate biopsy. In addition, one out of every four individuals with a serum PSA level between 4 and 10 ng/ml is likely to be affected with cancer. The risk of developing prostatic neoplasms increases if the serum PSA level exceeds 10 ng/ml. Considering the necessity of finding appropriate prostate cancer-specific markers for improved diagnosis, this study was designed to evaluate diagnostic value of *KLK2* and *KLK3* genes for prostate cancer.

MATERIALS AND METHODS

The study was carried out on 48 individuals who had been reffered to the urology unit of the Shahid Beheshti Hospital (Tehran, Iran). The subjects were divided into a prostate cancer group and a BPH group based on the results of bipsy and pathology tests. A control group comprising of 24 healthy individuals was also included in the study. In the prostate cancer group, patients who had been diagnosed for more than a year or had received hormone therapy, chemotherapy, radiation therapy or anti-cancer drugs were excluded from the study. In the BPH group, patients who had undergone open prostatectomy and were confirmed to be affected with BPH and not prostate cancer through histopathological analysis were excluded. A history of cancer, taking finasteride for longer than a month, taking anticancer drugs and detection of prostate cancer precursors (prostatic intraepithelial neoplasia) in prostate tissue were considered as the exclusion criteria for the BPH group. After obtaining written consent from all subjects, blood samples (2.5 ml) were taken and collected into EDTA-containing tubes and then immediately stored at 4 °C. All materials used in this study were purchased from Merck Co., Germany. RNA extraction and cDNA synthesis were performed in less than 2 hours after sampling. Before RNA extraction, lysed red blood cells (RBCs) and hemoglobin were removed from the samples. For this purpose, a lysis solution containing 10 mM Tris-HCl, sucrose, 5 mM $MgCl_2$ and 1% Triton X100 was prepared. According to the protocol, the lysis buffer was added with a 4-fold volume of the samples (10 ml). The solution was vortexed for 30-45 minutes and then centrifuged at 4000g for 20 minutes for complete lysis of RBCs. Supernatant was discarded and precipitate was washed with phosphate buffered saline. Finally, the extraction was done on the sediment (must be as transparent as possible) using a kit. Total RNA was extracted from blood using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purity of the extracted RNA was evaluated using a Nanodrop spectrophotometer

(Epoch, BioTek, USA) based on absorbance at 260 and 280 nm. Next, the integrity was checked by electrophoresis on 1% agarose gel and 1× Tris-Borate-EDTA. Later, cDNA was synthesized using 12 µl of the extracted RNA and then stored at -80 °C until reverse-transcription polymerase chain reaction (RT-PCR). Characteristics of the primers used in real-time RT-PCR are presented in table 1.

The 18s rRNA was used as a housekeeping gene to confirm the results of reverse transcription as well as the absence of contamination in samples prior to quantitative PCR. RT-PCR was performed using the CFX96 real time thermocycler system (BioRad, USA) and QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany).

Statistical analysis was performed using SPSS v.21 software. ANOVA and independent t-test were performed to compare data. A p-value of less than or equal to 0.05 was considered significant.

RESULTS

Mean age of subjects was 66.17 years (range: 47-87 & SD: 12.72) in the control group, 66.62 years (range: 58-79 and SD: 6.39) in the BPH group and 64 years (range: 56-75 & SD: 5.11) in the prostate cancer group. There was no significant difference between the groups in terms of age, height, weight, body mass index (BMI), history of dyspnea and smoking.

Expression of the reference gene (18srRNA) can be obtained relative to the measured CT value for each sample. In this study, the CT value of each sample was calculated by obtaining an average of duplicate assay (Table

Table 1- Variants of the genes under study and sequence of the primers designed by AlleleID V6 (Premier biosoft Co.)

KLK2	**NCBI accession number**	**Primer length**	**Amount used (µl)**	**GC %**	**T_m (°C)**	**Product length(bp)**
	variant 1: NM_005551.4 (2855) variant 2: NM_001002231.2 (2892) variant 3: NM_00125608.1 (2695)					
	Forward primer ATGGATGGGCACACTGT	**17**	**1**	**52.9**	**62.4**	**84**
	Reverse primer CCTGGCTATTCTTCTTTAG	**19**	**1**	**42.1**	**56.2**	
KLK3	**NCBI accession number**	**Primer length**	**Amount used (µl)**	**GC %**	**T_m (°C)**	**Product length (bp)**
	Variant 6: NM_001030050.1 Variant 1: NM_001648.2 Variant 4: NM_001030048.1 Variant 3: NM_001030047.1					
	Forward primer ACGTGGATTGGTGCTGC	**17**	**1**	**58.8**	**64.6**	**125**
	Reverse primer AGAACACCGCCGCAGA	**16**	**1**	**62.5**	**65.4**	
18s rRNA	**NCBI accession number**	**Primer length**	**Amount used (µl)**	**GC %**	**T_m (°C)**	**Product length (bp)**
	X03205 (1869)					
	Forward primer GTAACCCGTTGAACCCCATT	**20**	**1**	**50**	**64.5**	**151**
	Reverse primer CCATCCAATCGGTAGTAGCG	**20**	**1**	**55**	**64.2**	

Table 2- CT of the 18s rRNA gene in the blood of subjects

Group	Mean	Std. Deviation	Minimum	Maximum
Control	**25.6336**	**0.93006**	**24.22**	**27.23**
BPH	**25.3726**	**1.35195**	**22.20**	**28.09**
Prostate cancer	**25.5527**	**0.91593**	**24.27**	**27.42**
P-value (ANOVA)		**0.713**		

Table 3- The results of marker analysis and the mean CT in blood

Blood	Control	Mean CT	BPH	Mean CT	Prostate cancer	Mean CT
KLK2	**2/24**	**33.23**	**7/24**	**31.75**	**10/24**	**30.98**
KLK3	**21/24**	**28.80**	**19/24**	**25.65**	**20/24**	**27.33**

After performing real-time RT-PCR, the number of positive cases for expression of markers in each study group was determined (Table 3).

The *KLK2* was expressed in a small number of subjects. There was no significant difference between the three groups in terms of *KLK2* expression. However, mean CT values of *KLK2* differed significantly between the cancer and the control groups. In the case of *KLK3*, mean CT values did not differ significantly between the cancer and the control groups. However, there was a statistically significant difference in the mean CT value of *KLK3* between the BPH group and other groups (Table 4). Relative changes in the expression of the genes were assessed using the ΔCt method. As shown in table 5, there was no significant difference between the control and BPH

groups in terms of *KLK2* expression. However, the ΔCt of the *KLK3* gene differed significantly between the three study groups.
The ΔΔCt method indicated overexpression of the *KLK2* gene in the cancer group, which was 5.83-fold and 1.92-fold higher than in the control group and the BPH group, respectively (Table 6). Due to the low level of positive *KLK2*expression in the study groups, we found no significant correlation between the variables and this marker. There was no significant correlation between variables of age and the Gleason score and *KLK3* expression (Tables 7 and 8), but there was a significant relationship between the expression of this gene and BMI (Table 9).

Table 4- CT values related to *KLK2* and *KLK3* in the study groups

Variables	Control (1)		BPH (2)		Prostate cancer (3)		*P* value (t-test)			P-value (ANOVA)
	Mean	SD	Mean	SD	Mean	SD	(1) & (2)	(2) & (3)	(1) & (3)	
CT KLK2	33.23	0.07	31.75	1.60	30.98	1.03	0.254	0.245	0.014	0.083
CT KLK3	27.80	0.98	25.65	0.64	27.33	0.84	0.001	0.001	0.086	0.001

Table 5- Levels of the markers in patients with prostate cancer or BPH and in healthy controls

Variables	Control (1)		BPH (2)		PC (3)		*P* value (t-test)			P-value (ANOVA)
	Mean	SD	Mean	SD	Mean	SD	(1) & (2)	(2) & (3)	(1) & (3)	
ΔCT KLK2	7.47	0.80	6.64	2.64	5.16	1.09	0.830	0.131	0.019	0.153
ΔCT KLK3	3.16	1.24	0.28	1.60	1.81	1.20	0.001	0.001	0.001	0.001

Table 6- Results of ΔΔCt for each marker in the study groups

Marker	Group	Ct)ΔΔ2^-(
KLK2	Prostate cancer-Conrol	5.83
	BPH-Control	3.03
	Prostate cancer-BPH	1.92
KLK3	Prostate cancer-Conrol	4.95
	BPH-Control	1.77
	Prostate cancer-BPH	2.78

Table 7- Evaluation of *KLK3* expression in blood in different age groups

Age (years)	Mean ± SD	P-value
41-50	2.43±0.61	0.360
51-60	0.70±1.29	
61-70	1.08±1.87	
71-80	1.00±2.19	
81-90	1.56±0.00	

Table 8- Evaluation of *KLK3* expression in blood according to the Gleason score

Variable		Mean ± SD
Gleason score	< 7	27.29±1.04
	≥7	27.20±0.89
P-value		0.852

Table 9- Analysis of *KLK3* CT values in blood samples according to BMI

Variable		Mean ± SD
BMI (Kg/m^2)	< 23	26.81±1.23
	≥23	27.30±1.13
P-value		0.06

DISCUSSION

PSA has been used in prostatic neoplasms screening since the mid-1980s. Diagnosis of prostate cancer is currently based on the PSA test, followed by biopsy analysis levels, which limits the applicability of the test in case of initial confirmation of abnormality. However, a number of factors can affect PSA (17, 18). As mentioned by Thomson and colleagues, the PSA test is not specific nor sensitive enough for diagnosis of prostate

cancer (19). The test has low predictive values, which can produce notable false negative results on biopsies, which in turn might bring about the need for repeated tests and biopsy. The rate of negative biopsy is 60-75% in men with PSA levels of 3-10 ng/ml (20). In approximately 15% of men whose serum PSA levels are less than 4 ng/ml, prostate cancer is diagnosed through prostate biopsy.

It has been shown that PSA is present in normal breast tissues as well as in breast, colon, uterus, liver, kidney, adrenal and parotid tumor tissues (21). Therefore, increased level of PSA cannot be always attributed to prostate and prostate diseases. Based on the results of Ogawa et al., positive expression of PSA in peripheral blood is not correlated with age, preoperative serum PSA level and clinicopathological staging (22). Results of the studies on the association of PSA mRNA expression with prostate cancer have been controversial. In a study by Zhang et al., PSA expression in blood differed significantly between BPH patients, prostate cancer patients and those with metastatic prostatic neoplasms (23). In the present study, expression of the *KLK3* gene (ΔCT of KLK3) differed significantly between the three study groups. In a study by Ylikoski et al., although tumor grade was not correlated with PSA mRNA levels, there was a significant difference in PSA mRNA levels between the BPH group and prostate cancer patients, which suggests the suitability of the marker for discriminating BPH cases from patients with prostate cancer (24). Straub et al. demonstrated that preoperative PSA mRNA levels are significantly correlated with cancer stage (25). Inconsistent with this finding, two studies observed no correlation between preoperative PSA mRNA levels and cancer stage (26).

Zambon et al. found no difference in the PSA mRNA level between individuals with different Gleason scores (27). In the present expression in blood samples of patients with prostate cancer (P = 0.852). The *KLK2* gene codes for hK2, a serine protease that plays a direct role in the progression and metastasis of prostate cancer and may be a promising marker for diagnosis and discrimination of normal, BPH and cancer individuals. However, our results showed that the gene is expressed only in 26.5% of the subjects. Moreover, we only noted a significant difference in the expression of this gene between the controls and cancer patients. Few studies have investigated expression of this gene among individuals with prostatic neoplasms. In 2006, Juliana and colleagues evaluated *KLK2* and *KLK3* expression in peripheral blood samples and prostate cancer tissue samples. They concluded that the *KLK2* is a better marker for prostate cancer and its expression is related to cancer progression. Our results show that the expression of *KLK2* in peripheral blood is very low and not detectable in all individuals. Similarly, Juliana et al. detected the expression of this gene only in 14% of individuals with BPH and in 46.48% of patients with prostate cancer (28).

CONCLUSION

We demonstrate that analysis of *KLK2* expression is a favorable method of diagnosing prostate cancer and discriminating normal individuals from those with BPH or prostate cancer. The *KLK3* gene is highly overexpressed in individuals with BPH, which might indicate that this gene is not cancer-specific.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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